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An Approach to the Determination of the Relative Potencies of Chemical Agents During the Stages of Initiation and Promotion in Multistage Hepatocarcinogenesis in the Rat

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The potency of carcinogenic agents in eliciting neoplastic lesions has long been a concern of investigators in the field of oncology. This paper describes a method, based on quantitative stereologic calculations, to estimate the relative potency of chemicals as initiating and/or promoting agents. The parameters defined in this paper are: (a) Initiation index = no. foci induced × liver^{-1} × [mmole/kg body weight]$^{-1}$; and (b) Promotion index = V_f/V_c × mmole^{-1} × wk^{-1}. These parameters have been calculated for a number of chemical agents, based both on data from this laboratory and others published in the literature. Neither parameter varied significantly with the dose of two different initiating agents used in this study. The range of promotion indices extended over more than eight orders of magnitude, whereas that of the initiation indices was much less variable. Such parameters may be useful as quantitative estimates of the potency of hepatocarcinogenic agents not only in rodents, but potentially in quantitative risk estimations in the human.

Introduction

The multistage nature of carcinogenesis has now been demonstrated in a number of histogenetic systems in several species (*1–3*). Implicit in such a concept is that carcinogenic agents can act principally or exclusively at one or all of the definable stages in the carcinogenic process. Thus, complete and incomplete carcinogens have been defined as agents capable of acting at all stages of carcinogenesis or only at the stage of initiation, respectively (*3*). Promoting agents are considered to effect only the stage of promotion, although they may simulate the action of complete carcinogens through the promotion of spontaneously or fortuitously initiated cells (*3*).

A number of methods have been used to determine the relative carcinogenic potencies of chemical agents in rodents (*4*); however, few have attempted to quantitate the potency of an agent with respect to its action on a specific stage of carcinogenesis (*5*). Multistage carcinogenesis has been studied most extensively in the mouse epidermis and in the rat liver (*3,6*). In both of these systems, at least three distinct stages have been identified and, at least for the liver, definitions proposed (*7,8*). These stages have been termed initiation, promotion, and progression. Although studies on the skin have had a much longer history, the liver system offers the advantage, at least for quantitative studies, of the identification and enumeration of the early clonal progeny of initiated cells (*3,9*). This characteristic provides the process of multistage hepatocarcinogenesis in the rat with the potential for quantitating the potencies of agents in their action at each of the stages of hepatocarcinogenesis. Methods for determining such parameters for the stages of initiation and promotion in hepatocarcinogenesis in the rat are presented in this paper.

Methods

The data used for the derivation of the parameters described in this paper were obtained largely from studies carried out in this laboratory on the model described by us in 1978 (*10*). This model, like that of Peraino and his associates (*11*), allows for the administration of nonnecrogenic doses of an initiating agent, which may be a complete or incomplete carcinogen. For the most reproducible and distinctive quantitation of an agent's initiating potency, it is important that the dose of the initiating agent administered result in a yield of enzyme-altered foci (*11*), which can be accurately quantitated

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after their complete expression by administration of a promoting agent (*12*). The validity of this approach is supported by the finding from at least three different laboratories that each focus is clonal (*13–15*). The techniques for tissue preparation and quantitation of enzyme-altered foci have been previously described from this laboratory (*10*). A critical factor in such calculations with respect to the number of enzyme-altered foci is that quantitation in three rather than in two dimensions be employed, for reasons previously presented by this laboratory (*16*). On the other hand, for the determination of the parameter involving the potency of promoting agents, the area of the transections of enzyme-altered foci seen in two dimensions on a microscopic slide, given as a percentage of the total area of the slide (mm^2/cm^2), is identical with the volume percentage measurements in three dimensions.

A representative computer overlay of three serial sections stained respectively for γ- glutamyltranspeptidase (GGT), canalicular ATPase (ATPase), and glucose-6-phosphatase (G6Pase) is presented in Figure 1. It is from such preparations, with the use of computer or hand calculations, that one may determine the parameters described in this paper. Furthermore, although three markers are used in this figure, recent evidence (*17*) in confirmation of earlier studies (*10,18*) indicates that only two of these markers are needed to score more than 90% of the enzyme-altered foci, as long as one of the markers is GGT. Not used in this study is an even more efficient marker, the placental form of glutathione transferase (GST-P) (*19*). This marker is capable of scoring more than 90% of quantifiable enzyme-altered foci in rat liver as determined by quantitative stereologic methods (*17*). However, with some promoting agents, such as those inducing proliferation of hepatic peroxisomes (*20*), ATPase and G6Pase are needed in combination to score the same proportion, since many fewer nodules promoted by these agents express GST-P or GGT. Therefore, in order to score the maximal number of foci under all known conditions, GGT or, preferably, GST-P, together with either ATPase or G6Pase or a comparable marker, would be the preferred combination. Since identification of GST-P is carried out by an immunohistochemical procedure on acetone- or formalin-fixed tissues, another marker with the biological characteristics of either ATPase or a G6Pase, scored by similar techniques, would obviate the need for frozen-section technology.

Relative Potencies of Initiating and Promoting Agents—Initiation and Promotion Indices

A number of investigators (*5,21*) have classified some chemicals on the basis of their activity in promoting enzyme-altered foci and nodules after initiation with diethylnitrosamine (DEN) by quantitation of the num-

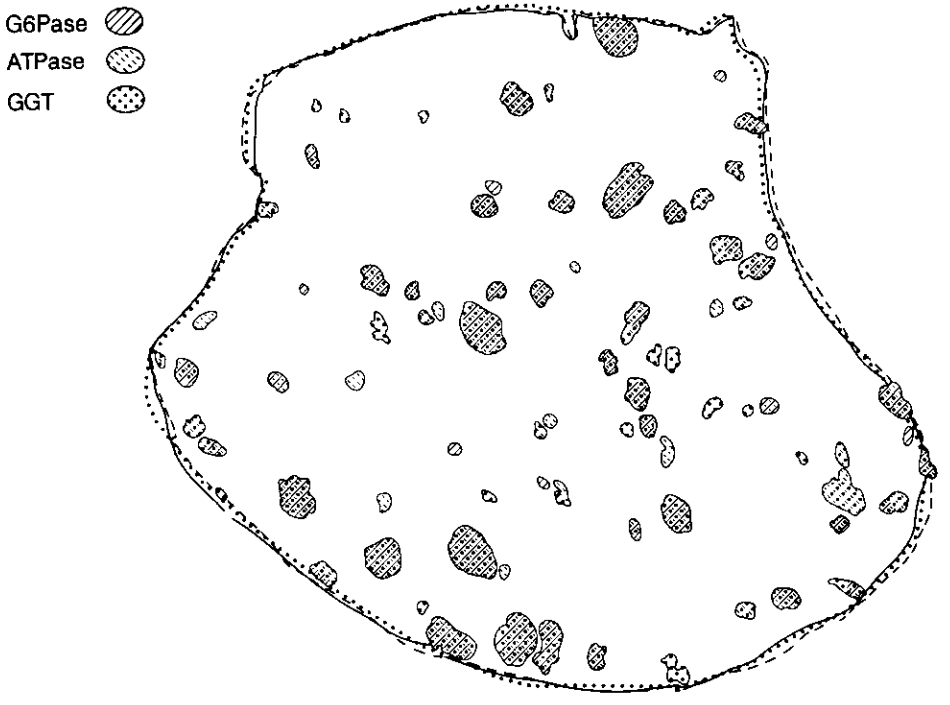

FIGURE 1. Artist's redrawing of a computer graphic plot of three serial frozen sections of liver from an animal treated with a single dose of diethylnitrosamine 24 hr following a 70% partial hepatectomy and subsequently placed on a diet containing 0.05% phenobarbital for 6 months (*10*). Each section is stained for one of the enzymes seen in the figure, which was generated by techniques previously described (*22*).

ber (in mm^2) of transections/cm^2 and area of the focal lesions as end points. We have developed a system based on the quantitative stereologic determination of the values of both number of enzyme-altered foci per liver and volume percentage occupied by these foci (*22*). As we have previously pointed out, such a three-dimensional calculation is critical in comparative studies, since it takes into account the differing sizes of the lesions that occur in most model systems (*16*).

We have based the calculations for determining the potency of a chemical as an initiating and/or promoting agent on the biological characteristics of each of these processes. As has been pointed out by a number of authors (*2,3,6,23,24*), initiation is a process occurring within a single cell, with this initiated cell conferring its altered state to all of its progeny. One definition of initiation is as follows:

> Initiation is that process occurring in a cell and resulting from the action of a chemical, physical, or biological agent that alters in an irreversible manner the heritable structure(s) of the cell. Such an initiated cell has the potential of developing into a clone of neoplastic cells (*8*).

In multistage hepatocarcinogenesis there is now ample evidence that each enzyme-altered focus is the clonal progeny of a single cell (*13–15*) that exhibits essentially all of the characteristics expected of an initiated cell population (*3,25*). Thus, for the purposes of these calculations, we have equated the number of enzyme-altered foci, as determined by three independent biochemical parameters (GGT^+, $ATPase^-$, and $G6Pase^-$ phenotypes) with the number of initiated cells. In this way one may relate the number of initiated cells to the dose of the initiating agent, since the latter is given only as a single dose in this and many other model systems of hepatocarcinogenesis (*26*). We have, therefore, defined the initiation index as follows:

$$\text{Initiation index} = \text{no. foci induced} \times \text{liver}^{-1} \times [\text{mmole/kg body weight}]^{-1}$$

The number of foci induced are those found in the liver of the animal that has been promoted with a maximally effective dose of promoting agent (*12*) for at least the time required to express all initiated cells quantifiable by the marker(s) used to score such clones minus the number of enzyme-altered foci occurring in animals not receiving the initiating agent but promoted with the same dose of promoting agent. This method thus corrects for levels of spontaneously or fortuitously initiated cells, whose formation is independent of the initiating agent administered. A calculation of the initiating index should be made from doses of the initiator that do not induce cell death or other severe toxicity, as every cell in the exposed population should have the same chance to become initiated.

The evaluation of the promoting potency of an agent is based on our knowledge of the nature of the biological effect of promoting agents as determined from experience in those systems *in vivo* in which multistage carcinogenesis is best understood, namely, the mouse epidermis and the rat liver. On the basis of these systems, one may define promotion as follows:

> Promotion is that stage in the natural history of neoplastic development which, if existent, is characterized by (a) the reversible expansion of the initiated cell population and (b) the reversible alteration of genetic expression (*8*).

In this definition, the emphasis is on reversibility, a biological characteristic of promotion in all known model systems of multistage hepatocarcinogenesis in the rat (*26*) and in most, if not all, such systems in the mouse epidermis (*27*). Numerous studies have demonstrated that promoting agents cause a selective increase in the numbers of the cells within enzyme-altered foci in rat liver (*9,26,27*), and all known promoting agents are known to alter gene expression in normal cells in a reversible manner (*3,6,28,29*). The phrase "if existent" is employed in this definition, since there are numerous examples, in chemical and physical as well as biological carcinogenesis, resulting from direct application of the carcinogenic agent with no demonstrable reversible stages. Presumably, under such conditions the stage of initiation is followed immediately by that of progression, the final irreversible stage of multistage carcinogenesis in which malignant neoplasms occur (*27,30*).

On the basis of such operational knowledge of the characteristics of promoting agents, we have defined the following parameter:

$$\text{Promotion index} = V_f hr / V_c \times \text{mmole}^{-1} \times \text{wk}^{-1}$$

where V_f is the total volume occupied by all the enzyme-altered foci in the liver of animals treated with the test agent, and V_c is the total volume of the enzyme-altered foci in the control animals, which have only been initiated. These volumes are expressed as a percentage of the total volume or weight of the liver. The millimoles of the promoting agent administered per week are determined either directly, if a measured amount of the agent is administered per day, week, or month, or as that consumed in an average food intake of 20 g/day (*31*). Since the volume occupied by the foci is directly related to cell number, the measurement of effectiveness of a promoting agent may, therefore, be related to its ability to stimulate selectively or to allow the replication of the promoter-dependent progeny of initiated cells (*32,33*). The correction for the volume percentage of enzyme-altered foci in animals that have only been initiated allows for the effect of uncontrolled endogenous or exogenous promoting agents in the experiment. Unfortunately, such a correction does not take into account any possible synergy between the test agent and uncontrolled promoting activities, such as dietary factors and endogenous hormones. Unlike the initiation index, which is determined after promotion for a period allowing the demonstration of the progeny of all initiated cells, the promotion index, although usually determined after 6 months of treatment with the promoting agent, may be determined at other time intervals during carcinogenesis as well. However, promotion indices must be calculated at levels of administration of the

promoting agent that exceed the threshold or no-effect levels of the agent and do not exceed the levels that result in a maximal effect (*12*). Therefore, it is important to determine the dose-response characteristics of the promoting agent prior to the evaluation of a promotion index for the compound.

Table 1 (*34–41*) shows some representative initiation and promotion indices taken both from studies reported in this paper and from those described earlier in the literature from this and other laboratories. In those values estimated from the literature and not from work in this laboratory, some assumptions had to be made, since the format of the experiments was somewhat different from those in the model used in this and other studies (*34,36*) from this laboratory. In the studies reported by Malvaldi et al. (*40*) on the promotive effects of thiobenzimide, the area of GGT^+ foci was used instead of the volume, but, as has been pointed out earlier (*16*), these two parameters are identical for the purposes of calculating promotion indices. Similarly, the promotion indices for 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) and *N*-2-fluorenylacetamide were calculated from the area of GGT^+ foci as presented in the work by Tsuda et al. (*41*). In this latter study, initiation was accomplished by a single IP dose of DEN (200 mg/kg). Two weeks later, rats were fed these agents in the diet for a period of 6 weeks, at which time the area of GGT^+ foci was determined. This study described many other compounds tested in a similar manner for which promotion indices could be calculated by the techniques described in this paper. The promotion index for 4-dimethylaminoazobenzene (DAB) was calculated from the volume percentage of those foci scored as GGT^+ in rats initiated by the administration of *N*-nitrosodiethanolamine in the drinking water (2000 ppm) for 6 weeks followed by a 2-week interval in which no carcinogen was administered, followed by the feeding of DAB in the diet (0.16%). In that study (*39*), foci were scored either as GGT^+ or $ATPase^-$, and the value in the table was that of GGT^+ enzyme-altered foci. The promotion index calculated from the $ATPase^-$ foci was 8.0.

It is of interest that, where values for initiation and promotion indices were calculated for the same compound in two different strains of rats, the values seen in the Sprague-Dawley animals were always higher than those of the F344 strain. On the other hand, when initiation indices for DEN and (7,12-dimethylbenz[a]anthracene (DMBA) are calculated over a range of doses (Table 2), the values do overlap, although animals of the Sprague-Dawley strain appear to exhibit higher values than those of the F344 strain. For both of these intitiating agents in any one strain, however, the values of the initiation indices within a single rat strain do not vary a great deal. Furthermore, as shown in Table 3, the promotion index is relatively stable over a wide range of doses of two different initiating agents, DMBA and DEN. The relatively high value seen at the 1.0 mg/kg dose of DEN is unexplained at the moment, but virtually all of the other values are relatively close to one another. Even in studies done in different laboratories of two closely related compounds, DAB and 3′-Me-DAB (Table 1), the calculated promotion indices from the available data are quite similar. As yet there are insufficient data to calculate promotion indices as a function of the concentration of the promoting agent administered, as only one or a few values have been available on the effective portion of the curve (*12*).

Discussion

At the present time, the determination of the carcinogenic activity of a chemical is basically a qualitative analysis. At the regulatory level of governmental agencies, no attempt is made to distinguish the action of chemical agents in the various stages of carcinogenesis; rather, all agents are assumed to be complete carcino-

Table 1. Relative potencies for initiation and promotion of hepatocarcinogenic agents in the rat.

Agent[a]	Initiation index[b]	Promotion index[b]	Reference
Proflavin	6.9×10^3	—	(*34*)
DMBA	1.6×10^5 (F344)[c]	—	(*35*)
DEN	7.2×10^5 (F344)	1:5 (F344)	(*35*)
	10.5×10^6 (S-D)[d]		(*35*)
Butylated hydroxytoluene	—	0.3 (S-D)	(*34*)
1′OH-Safrole	3.1×10^3	950	(*36*)
TCDD	0.0 (F344)	1.0×10^6 (F344)	(*35*)
		2.8×10^7 (S-D)	(*37*)
Wy 14,643	—	63	(*38*)
DAB	—	20	(*39*)
3′-Me-DAB	—	17	(*41*)
Thiobenzamide	—	60	(*40*)
Phenobarbital	0.0 (F344)	6 (F344)	(*34,35*)
		75 (S-D)	Unpublished observations
N-2-Fluorenylacetamide	—	927	(*41*)

[a] Abbreviations: DMBA, 7,12-dimethyl[a]anthracene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DAB, 4-dimethylaminoazobenzene; 3′-Me-DAB, 3′-methyl-4-dimethylaminoazobenzene.

[b] References to previously published studies are indicated in the reference column. The reader is referred to the text for details of the calculations.

[c] F344: Fischer F344/NHsdBR strain rats.

[d] S-D: Sprague-Dawley (SD)BR strain rats.

Table 2. Initiation indices as a function of dose of initiating agent in the rat.[a]

Initiating agent	Dose, mg/kg	Initiation index	
		F344	Sprague-Dawley
DEN	0.01	—	4.3×10^6
	0.1	—	6.0×10^6
	0.3	5.1×10^5	—
	1.0	1.7×10^6	1.4×10^6
	2.0	—	0.7×10^6
	3.0	4.4×10^5	—
	5.0	—	0.7×10^6
	10.0	2.2×10^5	—
DMBA	0.1	3.5×10^5	
	1.0	1.7×10^5	
	10.0	0.5×10^5	
	30.0	0.9×10^5	

[a] The format for the studies has been described (*35*). See footnotes of Table 1 for abbreviation key.

Table 3. Promotion index (PI) of phenobarbital in F344 female rats at various doses of the initiating agents DMBA and DEN.[a]

Initiating agent	Dose, mg/kg	Promotion index
DMBA	1.0	5
	10	10
	30	4
DEN	0.3	4
	1.0	26
	3.0	5
	10.0	6
	30.0	5

[a] See footnotes of Table 1 for procedural details and abbreviations.

gens if they exhibit any significant carcinogenic activity (*42,43*). Studies of multistage carcinogenesis in a number of different tissues in laboratory animals over the past several decades have demonstrated that such an assumption, in a significant number of instances, is not warranted (*1–3*). The reversible nature of the stage of tumor promotion, which has now been clearly shown in the best-studied multistage carcinogenesis models, those of mouse epidermis (*6,44*) and rat liver (*9,26*), demonstrates that no matter what mechanism is involved, the presence of a no-effect level is inherent in the characteristic of reversibility, and additivity may occur only in a relatively narrow window of the dose-response curve. Since risk estimations in the human always involve quantitative parameters, if animal studies are to be utilized in such determinations, then quantitation of the relative efficiencies of chemicals in animal systems should be very important for quantitative risk estimations across species lines.

Until recently, most methods for the determination of relative carcinogenic potencies of chemical agents related dose of the agent, either total dose or a dose rate, to some time function involving the yield or some fraction thereof of malignant neoplasms. An early proposal by Iball (*45*) related the carcinogenic potency of a chemical to the percentage of animals developing tumors divided by the latent period in days × 100. By this straightforward analysis, potent carcinogens usually induce many tumors in a relatively short time, whereas weak carcinogens induce fewer neoplasms, and then, only after a prolonged latent period. Barr's review (*4*) of various calculations used to determine carcinogenic potency refers to many different procedures that have been used in the past, both in experimental circumstances and also from regulatory agencies. Lutz and his associates (*46*) have developed equations relating the efficacy with which chemicals stimulate DNA synthesis and DNA binding in relation to their carcinogenic potencies. These calculations have been applied with some success to the prediction of carcinogenic potency for hepatocarcinogens in experimental situations. Parodi et al. (*47*) related the induction of preneoplastic nodules during hepatocarcinogenesis to the carcinogenic potencies of a variety of different compounds, most of which values were taken from studies in the literature.

Several studies, including some from our own laboratory (*12,18,48*), have demonstrated that the dose-response curves for initiation of enzyme-altered foci are different from those of promoting agents, in that the latter exhibit no-effect dose rates of promotion administration. Furthermore, a maximal effect of promoting agents is seen in the absence of toxicity, presumably resulting from the promotion of all foci developing from a finite number of initiated cells (*12*). Since it is impossible to prove unequivocally the presence or absence of a threshold under any circumstances involving multiple data points, despite the graphic appearance of the dose-response relationship, the ultimate interpretation of the data will depend on the presumed or observed mechanism of the agent in question. Initiation has been shown again and again to be an irreversible phenomenon in those systems in which this stage can be clearly distinguished (*3,6*). On the other hand, as described earlier, both in the liver and the skin systems in which the stage of promotion has been clearly defined and delineated, this stage is reversible. Thus, agents acting exclusively or even predominantly at this stage of carcinogenesis would be expected to exhibit threshold levels, as has been shown for numerous drugs and chemical agents exhibiting reversible effects in a variety of systems (*49*). The use of the number of focal lesions, each developing clonally from a presumed initiated cell, as a measure of the effectiveness of initiation is quite logical. The relative reproducibility of even the limited amount of data seen in this report would support this approach as a measure of the potency of a compound as an initiating agent in multistage hepatocarcinogenesis in the rat.

Promotion indices are apparently more variable than those for initiation (Table 1), as might be expected from the known environmental modulation of this stage (*2,3*). Despite this, promotion indices for the same or related compounds as studied in different laboratories are quite similar, as shown, for example, in Table 1 for DAB and 3′-Me-DAB (*39,41*). Another example is the similarity of the promotion index for phenobarbital as noted in Tables 1 and 3, and that of 7.0 as calculated from the data of Tsuda et al. (*41*) in the F344 strain from that laboratory. Although this report deals only with the

enumeration of enzyme-altered foci under the limits of identification used previously by this laboratory (*22*), recent studies (*50*) suggest that with different markers it may be possible to identify many more initiated cells. It will be of interest to determine in the future whether the inclusion of such presumably initiated hepatocytes in the calculations will relate to the potency of initiating agents in this system.

Unlike the initiation indices noted in Table 1, the range of promotion indices extends over almost eight orders of magnitude. Previous attempts to relate carcinogenic potencies also indicated an extremely wide range of potencies for a large number of chemicals (*51*). If one correlates these data with estimations from the literature, one conclusion is that the promotion index is more important than the initiation index for determining the overall carcinogenic potency of a chemical. It is also of interest that in all cases in which initiation or promotion indices were calculated for the same compound in two different strains of rats, the Sprague-Dawley strain always had a higher value. This finding is supported somewhat by the recent studies of Russell et al. (*52*), who compared initiation by DEN and promotion by phenobarbital in the same two strains of animals. Their studies also showed a greater sensitivity to these two compounds in the Sprague-Dawley strain. Further studies are needed to determine exactly how consistent promotion indices are under various experimental conditions of diet, sex, age, etc., since all of these factors have been shown to affect the stage of promotion in multistage carcinogenesis (*2,3,23,24*).

Clearly, the dose response affects the promotion index, because no valid index can be calculated below the threshold level of the agent, and calculation of promotion indices after a maximal effect of the promoting agent has been obtained will lead to spuriously low results. Thus, meaningful promotion indices that can be compared from laboratory to laboratory and even from experiment to experiment must be calculated on the relatively linear portion of the dose-response curve. Similarly, initiation indices must be calculated from values on the increasing, relatively linear portion of the dose-response curve (*48*) in order to eliminate possible artifacts due to cell death, toxicity, etc.

Although the potency parameters proposed in this paper are limited to hepatocarcinogenic agents, other systems are now being developed in which the clonal progeny of initiated cells may be quantitatively determined and in which quantitation of the total promoted cell population is possible. Analogous calculations of potencies may already be applicable, in part, at least, to such tissues as the epidermis (*53*), pancreas (*54*), lung (*55*), thyroid (*56,57*), brain (*58,59*), and mammary gland (*60,61*). Such quantitative calculations, based on a knowledge of the characteristics of these two stages of carcinogenesis, may be helpful in determining quantitative parameters useful in risk assessment in the human being.

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